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Determination of the cyanide metabolite 2-aminothiazoline-4-carboxylic acid in urine and plasma by gas chromatography–mass spectrometry

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Abstract

The cyanide metabolite 2-aminothiazoline-4-carboxylic acid (ATCA) is a promising biomarker for cyanide exposure because of its stability and the limitations of direct determination of cyanide and more abundant cyanide metabolites. A simple, sensitive, and specific method based on derivatization and subsequent gas chromatography–mass spectrometry (GC–MS) analysis was developed for the identification and quantification of ATCA in synthetic urine and swine plasma. The urine and plasma samples were spiked with an internal standard (ATCA- d_2), diluted, and acidified. The resulting solution was subjected to solid phase extraction on a mixed-mode cation exchange column. After elution and evaporation of the solvent, a silylating agent was used to derivatize the ATCA. Quantification of the derivatized ATCA was accomplished on a gas chromatograph with a mass selective detector. The current method produced a coefficient of variation of less than 6% (intra- and interassay) for two sets of quality control (QC) standards and a detection limit of 25 ng/ml. The applicability of the method was evaluated by determination of elevated levels of ATCA in human urine of smokers in relation to non-smokers for both males and females.

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1. Introduction

Exposure to cyanide can occur by a number of scenarios, including the use of cyanide as a chemical warfare agent (CWA) [1–5]. The analysis of biological matrices, specifically urine and blood, to verify exposure to cyanide is important to determine past exposure to cyanide [1,5]. Due to its volatility, hydrogen cyanide rapidly dissipates from open areas. Therefore, biological fluids of persons or animals exposed to hydrogen cyanide may be the only repository of evidence that cyanide was used as a CWA. As

seen in Fig. 1, three major markers of cyanide exposure appear in the blood or urine of exposed individuals: cyanide ion (CN^-), thiocyanate (SCN^-), and 2-aminothiazoline-4-carboxylic acid (ATCA) or its tautomer 2-iminothiazolidine-4-carboxylic acid (ITCA).

Although many sensitive methods exist for analysis of cyanide ion [6], they all suffer from the practical limitations that stem from cyanide's volatility and nucleophilic nature. Under normal physiological conditions, cyanide (pK_a 9.2) is protonated. This protonated form of cyanide is extremely volatile [5,7], which contributes to its elimination from analytical samples. The nucleophilic nature of the non-protonated form of cyanide also contributes to its elimination from biological media. Therefore, depending on the route

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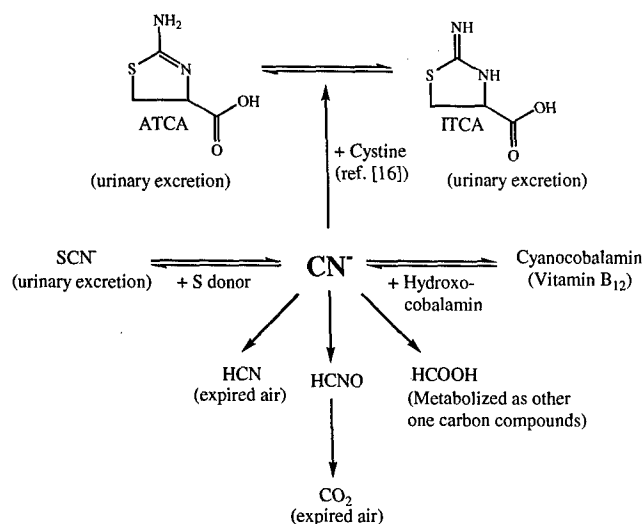


Fig. 1. Cyanide metabolism and major modes of release. Adapted from Baskin et al. [6]. A proposed mechanism for the formation of ATCA from the reaction of cyanide with cystine is presented by Nagasawa et al. [16].

and duration of exposure, cyanide is typically eliminated from blood within 20 min post-exposure [5,7,8]. In addition to rapidly decreasing concentrations in biological fluids, cyanide sometimes forms as an artifact of storage conditions in a variety of biological samples, including blood [9–11]. Cyanide exhibits variable concentrations in biological fluids depending on the route of exposure (e.g., oral versus inhalation) [12] and has significant endogenous (inherently present) concentrations. Some sources of endogenous cyanide include normal metabolism of amino acids within the body along with external sources such as smoking, inhalation of smoke from fires, and some types of food [5]. Because of these limitations, analytical determination of cyanide metabolites is generally more advantageous to direct cyanide determination. It should be noted that the metabolites of cyanide should also exhibit significant endogenous concentrations, originating from the same sources of endogenous cyanide.

Thiocyanate is the major metabolite of cyanide, and a number of sensitive methods are available for its measurement in biological fluids [6]. Thiocyanate has many advantages as a marker for cyanide exposure. It is non-volatile and can be analyzed in blood, urine, and saliva. However, thiocyanate does have some disadvantages as an indicator of cyanide exposure. Ballantyne [10] found that analytical recovery of thiocyanate from whole blood is not quantitative and thiocyanate concentrations in blood varied inconsistently during storage at various temperatures over a period of 2 weeks. Isom and Baskin [13] also noted that thiocyanate may be formed by other modes of metabolism besides cyanide intoxication or metabolism.

While there are numerous sensitive analytical techniques to determine both cyanide and thiocyanate in biological fluids, very few techniques exist to analyze for ATCA. In fact, the liquid chromatographic analytical technique of Lundquist et al. [14] and the spectrophotometric method of Wood et al. [15]

are the only available methods for determination of ATCA in biological fluids. ATCA accounts for approximately 20% of cyanide metabolism and increases as exposure increases [5]. ATCA is formed when cyanide reacts with L-cystine through a proposed intermediate, β -thiocyanoalanine, that is subsequently transformed to ATCA (Fig. 1) [16]. ATCA may be present as a tautomer between itself and 2-iminothiazolidine-4-carboxylic acid (ITCA). The structure of ATCA resembles a cyclic amino acid and appears to have the same general properties as an amino acid with similar functional groups. ATCA may be an excellent marker for indicating cyanide exposure primarily because of its stability. It is stable for months in biological samples at freezing and ambient temperatures [14]. The structure of ATCA also lends itself to simple but extremely effective sample preparation and derivatization.

ATCA represents an alternative metabolite of cyanide exposure, and a simple assay for ATCA may circumvent the disadvantages of cyanide and thiocyanate analysis. Therefore, the objective of this study was to develop a novel analytical method to determine ATCA concentrations in urine and plasma for the purpose of simple and accurate verification of exposure to cyanide.

2. Experimental

2.1. Reagents and standards

All chemicals and solvents were at least HPLC grade. ATCA (L-2-aminothiazoline-4-carboxylic acid) was obtained from Chem-Impex International (Wood Dale, IL, USA). A standard stock solution (100 $\mu\text{g}/\text{ml}$) was prepared in 0.1 M HCl and used throughout the current study. An aqueous ammonium hydroxide solution (approximately 50%) was used to prepare elution solvents. Oasis[®] MCX (mixed-mode cation exchange) columns were obtained from Waters Corporation (Milford, MA, USA). The derivatizing agent *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide (MSTFA) was purchased from Pierce Chemical Company (Rockford, IL, USA). All solvents were purchased from Sigma-Aldrich (St. Louis, MO, USA). Deuterated ATCA (ATCA- d_2) was obtained from the lab of Dr. Herbert T. Nagasawa (Department of Veterans Affairs Medical Center, Minneapolis, MN, USA) prepared by reaction of deuterated L-cysteine (3,3- d_2) with cyanamide [16].

2.2. Biological fluids

Synthetic urine and swine (*Sus scrofa*) plasma were used to optimize analytical methodologies and sample preparation techniques. The use of synthetic urine as a sample matrix was necessary due to endogenous concentrations of ATCA in urine. The synthetic urine was prepared as a 1 kg stock solution according to Inn et al. [17] with chemicals obtained from Sigma-Aldrich (St. Louis, MO, USA), TCI (Tokyo Chemical International, Tokyo, Japan), and Acros Organics (Mor-

ris Plains, NJ, USA). Swine plasma (non-sterile with EDTA anti-coagulant) was purchased from Pel-Freez Biologicals (Rogers, AR, USA).

Urine and blood samples from human (*Homo sapiens*) subjects, age 21–50 years, were used to evaluate the applicability of the analytical method. Urine samples were used to determine if a significant difference in ATCA concentration could be found in the urine of smokers relative to non-smokers. Smokers consume small amounts of cyanide from the inhalation of cigarette smoke. Therefore, ATCA concentrations in the urine of smokers should be elevated relative to non-smokers. The number of samples used for this study totaled 40 with 23 males and 17 females. Volunteers donated urine in the morning in sterile urinalysis cups that were subsequently stored at 4 °C until analyzed. All samples were analyzed less than 14 days after they were gathered. No personal information was recorded from the volunteer donors except their gender and whether they were a smoker. Plasma was obtained from male non-smokers to determine whether endogenous levels of ATCA could be detected by the current method. Plasma from smokers was not analyzed for the current study.

2.3. Sample preparation

Urine samples were diluted, acidified, and passed through a mixed-mode cation exchange column to prepare the samples for gas chromatography–mass spectrometry (GC–MS) analysis. Specifically, 100 μ L of sample (spiked synthetic urine, human urine, spiked swine plasma, or human plasma) was diluted with 900 μ L of 0.1 M HCl to ensure the ATCA was positively charged (pH < 1). ATCA- d_2 (100 ng/ml) was added to the diluted sample as an internal standard. This diluted sample was then aspirated through an MCX column, after conditioning with successive washes of 1 ml each methanol and H₂O. The column was then washed successively with 1 ml each of 0.1 M HCl and 100% methanol. The ATCA was eluted from the columns using 1 ml of CH₃OH:NH₄OH:H₂O (50:25:25) into 2 ml micro-centrifuge tubes. HCl (200 μ L of 0.1 M) was added to the centrifuge tubes to decrease the pH of the sample (pH < 11) prior to drying (the ring in ATCA may open under heat and basic conditions [14]). The sample was dried completely in a centrifugal evaporation system (Labconco, Kansas City, MO, USA) at 40 °C. The dried samples were derivatized with 150 μ L of 30% MSTFA in hexane for 60 min at 50 °C in capped centrifuge tubes to produce the trimethylsilyl (TMS) derivative of ATCA as illustrated in Fig. 2. The sample was then transferred with borosilicate transfer pipets to GC–MS autosampler vials fitted with 200 μ L deactivated glass inserts.

2.4. Gas chromatography–mass spectrometry

Analysis was performed on an Agilent GC–MS system consisting of a 6890N Series II gas chromatograph, a 5973 series mass-selective detector, and a 7683 auto-sampler. A DB-

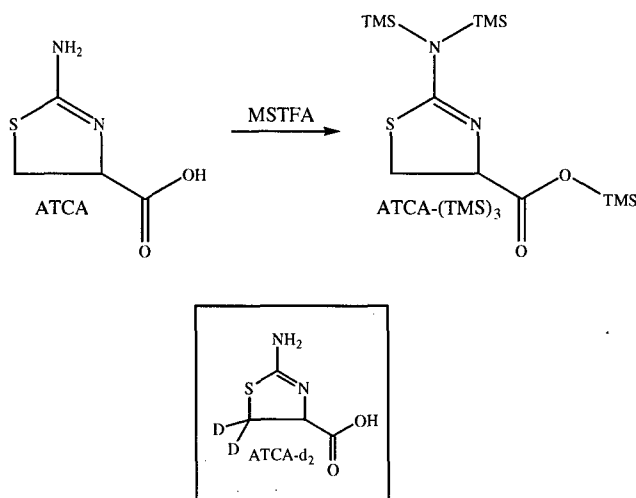


Fig. 2. Derivatization reaction of ATCA with MSTFA. TMS, trimethylsilyl group Si(CH₃)₃. Inset: ATCA- d_2 chemical structure.

5 MS bonded phase column (30 m \times 0.25 mm I.D., 0.25 μ m film thickness; J&W Scientific, Folsom, CA) was used with helium as the carrier gas at a flow rate of 0.8 ml/min and column head pressure of 52.4 kPa (7.6 psi). The auto-sampler introduced a 1 μ L sample into an injection port with a total inlet flow of 54 ml/min. The injection port was held at 290 °C and contained an Agilent inlet liner of deactivated borosilicate single-taper with glass wool packing. The purge flow was initiated at 1 min with a flow of 50 ml/min. The GC oven temperature was initially held at 100 °C for 1 min, then elevated at a rate of 15 °C/min up to 230 °C. The gradient was then increased to 30 °C/min up to 300 °C where it was held constant for 1 min. This gradient resulted in an overall run time of 13 min with ATCA-(TMS)₃ eluting at approximately 8.76 min. The GC was interfaced with a mass-selective detector with the transfer line held at 265 °C. Fragmentation of the sample was accomplished through electron impact with selected ion monitoring (SIM) mode for monitoring abundant ions of ATCA (m/z 245, 347, and 362) and ATCA- d_2 (m/z 349, and 364) with a dwell time of 100 ms each. The MS conditions were as follows: ion source pressure 2.0 mPa (1.5×10^{-5} Torr), source temperature 230 °C, quadrupole temperature 150 °C, electron energy 70 eV, electron emission current 34.6 μ A, and electron multiplier voltage +400 relative to the autotune setting.

2.5. Calibration and quantification

Calibration and quality control (QC) standards were prepared from the stock ATCA solution in the range of 10–2000 ng/ml by diluting in synthetic urine. Calibration standards were prepared with concentrations of 10, 20, 50, 100, 200, 500, 1000, and 2000 ng/ml. The 2000 ng/ml standard was found to be above the upper limit of quantification (ULOQ) for the method, and the 10 and 20 ng/ml standards were below the lower limit of quantification (LLOQ). A percent relative standard deviation of less than 10% and a percent

deviation of less than $\pm 20\%$ back-calculated from the nominal concentration for each calibration standard were used as criteria for determining the ULOQ, LLOQ, and the linear dynamic range. QC standards ($n = 5$) were prepared at 150 ng/ml and 800 ng/ml. *Intraassay* studies were performed by analysis of five replicate QC standards at 150 and 800 ng/ml during 1 day and *interassay* studies were performed by analysis of these same quintuplicate QC standards on 3 different days (within 1 week). The intraassay studies were designed to determine the stability of the method for a single analysis (i.e., construct a calibration curve and analyze the QC standards to validate the method for that analysis). The interassay studies were designed to evaluate the stability of the method for a number of days of analysis (i.e., construct three calibration curves on 3 separate days and analyze the same QC standards for each calibration curve to validate the method for multiple analyses).

2.6. ATCA stability

All stability experiments were performed on samples of 1000 ng/ml ATCA in synthetic urine. Short-term stability experiments included evaluation of the stability of ATCA during multiple freeze-thaw cycles, stability of ATCA on the bench-top, and stability of TMS-derivatized ATCA in the autosampler. Freeze-thaw stability was determined by completing five freeze-thaw cycles with analysis of ATCA after each individual cycle. For verification of bench-top stability, a urine sample was spiked with ATCA and allowed to stand at room temperature. The spiked sample was analyzed at 0, 1, 2, 4, 8, and 24 h. For the autosampler stability experiment, eight sets of samples (each in triplicate) were derivatized, placed in the autosampler, and analyzed at approximately 1, 4, 7, 9, 12, 15, 18, and 20 h post-derivatization to determine the stability of the TMS derivatized ATCA.

Experiments were also undertaken to determine the long-term stability of ATCA under typical storage conditions. Five long-term stability samples were prepared with a range of normal urinary pH values (4, 6, and 8) [18] and stored at 4, -20 , and -70°C . Three aliquots of these samples were analyzed prior to storage. The samples were then stored for 3 months with analysis at the end of each month. Three replicates of each sample were analyzed for each sampling period. For example, at the end of the 2nd month, samples were removed from storage and allowed to thaw if necessary, three aliquots of each sample were removed and analyzed, and the samples were placed back into storage.

2.7. Assay selectivity and recovery

The selectivity of the assay was determined by comparison of multiple blank samples of synthetic urine and swine plasma to determine the potential for interfering (unresolved) background peaks that co-elute with the ATCA-(TMS)₃. To determine recovery, an aliquot of ATCA was spiked into synthetic urine or swine plasma for a final concentration of 1000 ng/ml.

The sample was then prepared and analyzed as above. The recovery of ATCA was calculated as a percentage by dividing the recovered ATCA concentration by the nominal concentration added to the urine or plasma.

2.8. Data analysis

Calibration was performed by plotting peak area ratios of the characteristic ions of ATCA (m/z 362) and the internal standard, ATCA-d₂ (m/z 364) as a function of the spiked ATCA concentration. A weighted ($1/x^2$) linear fit was employed for all calibration curves. Precision was calculated as a %CV by dividing the standard deviation of the peak area ratios by the mean peak area ratio. The accuracy was calculated as a percentage by dividing the mean calculated concentration by the intended concentration. Precision and accuracy were calculated for each set of QC standards (for intra- and interassay evaluation). Data from long- and short-term stability experiments were handled by determination of ATCA concentrations as a percentage of a positive control. Stability is represented by a percent difference from the positive control. For the stability experiments, positive controls were freshly prepared control samples with the same nominal concentration of ATCA as the sample to be analyzed. Comparison of the urine samples for significant differences in the ATCA concentrations of smokers compared with non-smokers was accomplished by a two-tailed *t*-test. Comparison of the four groups of volunteers (female non-smoker, female smoker, male non-smoker, and male smoker) was accomplished by an analysis of variance and the Bonferroni, Tukey, and Newman-Keuls multiple range tests to identify significant differences between the identified subsets of volunteers.

3. Results and discussion

3.1. Sample preparation and GC-MS analysis

ATCA has a basic amine moiety and an acidic carboxylic acid moiety (Fig. 1). The amine is positively charged at neutral to low pH values and the carboxylic acid is negatively charged at medium to high pH values. Therefore, either the amine, the carboxylic acid, or both are charged at all aqueous pH values. The sample preparation scheme was developed to take advantage of the positively charged amine at low pH values. Samples were acidified and mixed-mode cation exchange columns were used to electrostatically bind the positively charged ATCA on the column while allowing neutral and negatively charged compounds through the column. A basic solvent was used to neutralize the amine and release it from the cationic sorbent.

After sample preparation, ATCA must be derivatized to analyze by GC-MS because it is non-volatile. ATCA was derivatized with MSTFA, which can react with primary and secondary amines and carboxylic acid moieties, replacing

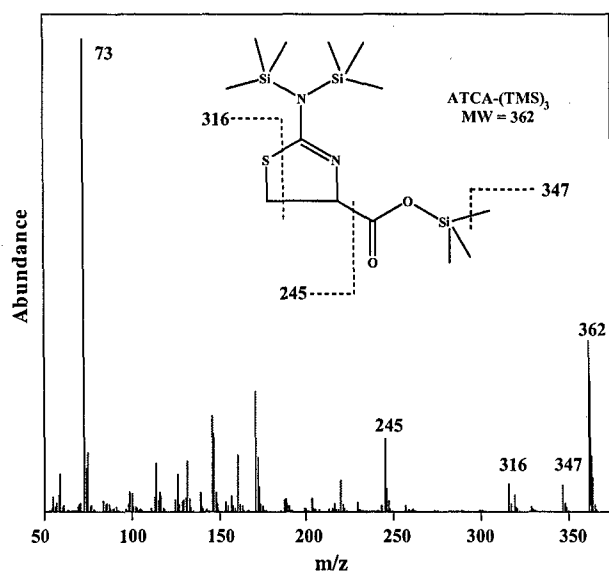


Fig. 3. Electron impact mass spectrum of trimethylsilyl (TMS) derivatized ATCA and abundant ions. Ions with a mass-to-charge ratio of 245, 347, and 362 were monitored to analyze for ATCA in urine samples. The large 73 *m/z* ion above corresponds to one TMS fragment. Inset: Structure of derivatized ATCA with abundant fragments indicated.

exchangeable hydrogens with TMS groups. Fig. 2 illustrates the chemical structure of ATCA and the most likely product of derivatization with MSTFA. Inset is the structure of the internal standard (ATCA-d₂).

Fig. 3 illustrates the mass spectra of derivatized ATCA. The mass-to-charge ratio of 362 corresponds to the molecular ion of the ATCA-(TMS)₃. The probable fragmentation of the ATCA-(TMS)₃ derivative (corresponding to ions 347, 316, and 245 *m/z*) is also illustrated. Fig. 4 shows a representative chromatograph of both a blank synthetic urine sample (0 ng/ml ATCA, lower trace), a spiked synthetic urine sample (1000 ng/ml ATCA, upper trace), and a human urine sample (middle trace). Fig. 5 shows a representative chromatograph of blank human plasma (0 ng/ml ATCA, lower trace) and a spiked human plasma sample (1000 ng/ml ATCA, upper trace).

3.2. Linearity and dynamic range

Calibration curves were constructed in the range of 50–1000 ng/ml ATCA with five concentrations, each in triplicate, in addition to five blank samples (0 ng/ml ATCA, nominal concentration) for 3 separate days. The data were best described by a weighted ($1/x^2$) linear least squares fit of the calibration samples. The best model was determined by analysis of the weighted sum-of-squares and the absolute relative error for each model. The dynamic range for the method was 50–1000 ng/ml ATCA, and the limit of detection was found to be 25 ng/ml with 3 separate days of validation when calculated at a signal-to-noise ratio of 3 ($n=5$ for each day).

It should be noted that an internal standard with only two deuterium atoms may result in overestimation of internal

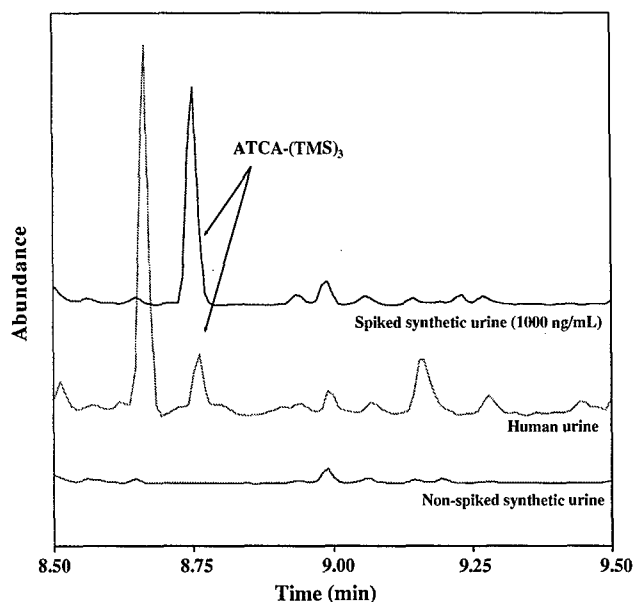


Fig. 4. Chromatographs of non-spiked synthetic urine (lower trace), 1000 ng/ml ATCA spiked into synthetic urine (upper trace), and non-spiked human urine (middle trace). The human urine was sampled from a male non-smoker. The human urine was sampled from a male non-smoker with a peak area corresponding to an ATCA concentration of 225 ng/ml. The chromatographs represent the sum of the individual ions gathered in SIM mode after sample preparation of the urine samples with SPE and derivatization. No internal standard was added to the urine samples prior to analysis. ATCA-(TMS)₃ elutes at approximately 8.76 min.

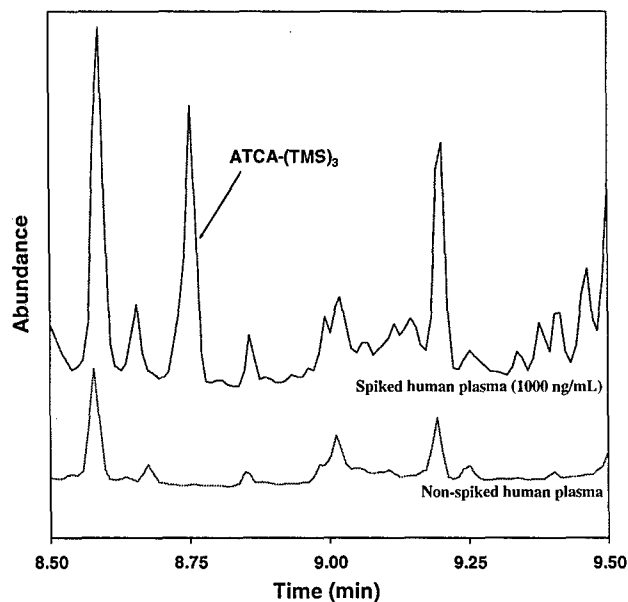


Fig. 5. Chromatographs of non-spiked human plasma (lower trace) and spiked human plasma (upper trace). The plasma was sampled from a male non-smoker. The chromatographs represent the sum of the individual ions gathered in SIM mode after sample preparation of the urine samples with SPE and derivatization. No internal standard was added to the plasma samples prior to analysis. ATCA-(TMS)₃ elutes at approximately 8.76 min.

Table 1
Quality control data for ATCA analysis by GC–MS in synthetic urine

Nominal concentration (ng/ml)	Intraassay accuracy (%) ^a	Interassay accuracy (%) ^b	Intraassay precision (%CV) ^a	Interassay precision (%CV) ^b
150	105–100	103	<4.1	5.3
800	99.0–97.4	98.0	<3.0	5.8

^a Mean of 1 day of validation ($n = 5$ for each day; overall range is for 3 separate days).

^b Mean of 3 days of validation ($n = 15$).

standard peak areas, particularly when analyte concentrations are large [19]. This potential problem was accounted for by developing a calibration curve that excluded calibrators with a percent deviation of less than $\pm 20\%$ from the nominal concentration. If the internal standard area is overestimated, the ratio of analyte to internal standard would decrease, and therefore increase the percent deviation of the calibrator from its nominal concentration. If the overestimation becomes large enough for a particular concentration, that calibration standard would be excluded from the curve. For practical purposes, if analyte concentrations are above the ULOQ of the calibration curve (i.e., overestimation of the internal standard concentration is occurring), dilution of the sample or use of a smaller sample volume and reanalysis could be performed. Alternatively, the curve could be reconstructed with an increased internal standard concentration.

3.3. Accuracy and precision

The intra- and interassay accuracy and precision are reported in Table 1 for the two QC samples. The accuracy and precision reported in Table 1 indicate that the method is extremely precise and also accurate for the concentrations tested. The ranges of percent relative standard deviation of the calibrators for the ULOQ (1000 ng/ml) and LLOQ (50 ng/ml) were 1.5–2.0% and 0.42–8.0%, respectively ($n = 3$ per day, for 3 days; data not shown). The ranges of percent deviation back-calculated from the nominal concentration of the calibration standards for the ULOQ and LLOQ were 92–94% and 91–96%, respectively ($n = 3$ per day, for 3 days; data not shown).

3.4. Assay selectivity and recovery

Assay selectivity is demonstrated by the absence of interfering peaks in the total ion chromatograph. In Fig. 4, the ATCA-(TMS)₃ peak elutes at approximately 8.76 min and the blank synthetic urine has little background signal over the time where ATCA-(TMS)₃ elutes from the column. The ATCA-(TMS)₃ peak also elutes during a region where there is little background signal from the plasma (Fig. 5). Therefore, the selectivity of the method was adequate for both plasma and urine. Over twenty samples of blank (non-spiked) synthetic urine were analyzed to demonstrate assay specificity and selectivity. Although surrogate samples were used for

urine, direct experimental evidence of excellent selectivity in human plasma was gathered (Fig. 5).

The recovery of ATCA was probed in spiked plasma and urine samples. The recovery of ATCA was 84% ($n = 6$) and 100% ($n = 6$) for synthetic urine and swine plasma samples, respectively. No matrix effects were observed except when the post solid-phase extraction column addition of 0.1 M HCl was omitted from the analytical procedure. When this step was omitted, the absolute ATCA-(TMS)₃ signal was decreased. It should be noted that the internal standard did correct for this effect. It should also be noted, that the selectivity and recovery of ATCA from human urine or plasma may be different than that found during experiments using synthetic urine and the swine plasma because they are surrogate matrices and can only approximate human urine and plasma.

3.5. Short- and long-term stability

Both the short- and long-term stability of ATCA were found to be excellent, with Table 2 illustrating that none of the samples deviated from the control by more than $\pm 9.3\%$ over the course of all stability experiments. The stability of ATCA was validated in synthetic urine for 24 h on the benchtop, through 5 freeze-thaw cycles, and for 3 months at 4, -20 , and -70°C and pH values of 4, 6, and 8. Stability of the TMS derivatized ATCA was also demonstrated by analysis of the derivative with increasing periods of storage at room temperature after derivatization. The largest deviation from the

Table 2
Short- and long-term stability of ATCA in synthetic urine

Stability	Stability experiment	Greatest deviation from control (%) ^a
Short-term ^b	Autosampler ^c	2.8
	Benchtop	4.0
	Freeze-thaw	8.9
Long-term ^b	pH 4, 4°C	9.3
	pH 6, 4°C	5.6
	pH 8, 4°C	-6.8
	pH 6, -20°C	-5.8
	pH 6, -70°C	4.4

^a The deviation of each individual sample from the positive control was calculated and the largest deviation is reported.

^b Samples for long-term stability were analyzed for 3 months. Short-term stability was determined over 20 h for autosampler stability experiments, 24 h for benchtop stability experiments, and over five freeze-thaw cycles.

^c Stability of TMS derivatized ATCA at room temperature.

Table 3

Breakdown of human urine samples of smokers and non-smokers analyzed for ATCA in this study

Urine samples	Number of samples			ATCA concentration (ng/ml)		
	Male	Female	Total	Male	Female	Total
Smokers	8	11	19	115 ± 36	319 ± 284	233 ± 237
Non-smokers	15	6	21	87 ± 46	80 ± 53	85 ± 47

control occurred during the freeze-thaw and the long-term stability experiments. It should be noted that the largest deviation for the long-term stability samples occurred in the 2nd month, whereas in the 3rd month, all samples were found to deviate by less than 5% of the control value. Also, the largest deviation during the freeze-thaw experiments occurred on the second freeze-thaw cycle compared with the third through fifth freeze-thaw cycle of less than 5%. This indicates that the deviation from the control is not associated with instability in ATCA concentrations, but more consistent with random error in the overall analysis of ATCA.

3.6. Analysis of ATCA in human urine and blood

Applicability of the current analytical method for ATCA analysis was achieved by analyzing human urine to determine concentrations of ATCA in non-smoking and smoking human volunteers. Prior attempts to determine ATCA concentrations in non-smokers have failed to determine concentrations above the detection limit of the analytical method [14]. Fig. 4 shows the GC–MS chromatograph of urine in a male non-smoker without ATCA-d₂. A significant ATCA-(TMS)₃ peak is evident in the urine of this subject and is absent from the non-spiked synthetic urine, which indicates that endogenous concentrations of ATCA can be determined by the analytical method presented.

The urine of forty human volunteers was used to determine concentrations of ATCA in male (23 total) and female (17 total) smokers and non-smokers. Reported in Table 3 is a breakdown of the number of volunteers by smoker or non-smoker and male or female. The mean and standard deviation of ATCA concentrations analyzed in urine for each group are also reported in Table 3. It is clear that the mean ATCA concentration increases for smokers as opposed to non-smokers in each individual group. The difference in ATCA concentrations between smokers and non-smokers were tested for significance with a two-tailed *t*-test. The difference was significant to the 99% confidence interval for the pooled data (all samples, only differentiated by smoker or non-smoker). An analysis of variance was performed on the data, broken down into female non-smoker, female smoker, male non-smoker, and male smoker to determine if there were significant differences between the groups indicated. It was determined that there were significant differences between male smokers compared with female smokers, male non-smokers compared with female smokers, and female non-smokers compared with female smokers. The ability of the analytical method to determine a significantly elevated concentration of ATCA in

the urine of smokers relative to non-smokers (for the pooled data) indicates that the analytical method presented will be able to determine past exposure to higher doses of cyanide.

Although the analytical method can determine significant differences between urinary ATCA concentrations of smokers versus non-smokers, the large concentration of endogenous ATCA found in urine may present a potential problem for using this analytical technique to verify exposure to cyanide. Although this is a potential problem, it is mitigated by the fact that other markers of cyanide exposure also suffer from this problem, and endogenous concentrations for other chemical agent markers have been addressed in the past [20,21]. Endogenous concentrations of ATCA in human plasma were found to be below the detection limit for the method presented (Fig. 5), possibly allowing for the definitive verification of exposure to cyanide.

4. Conclusions and perspectives

The current GC–MS method to determine ATCA in urine and plasma is a sensitive and precise analytical method that shows great promise to augment or replace current analytical methods to determine past exposure to cyanide. This study indicates that the method presented can be used with human urine and plasma samples and can detect endogenous levels of ATCA in the urine of non-smokers.

Future studies include a broad analysis of ATCA in human urine of smokers and non-smokers to further evaluate the analytical method's ability to determine ATCA in human urine samples and to establish approximate endogenous concentrations of ATCA in human urine. These endogenous concentrations will be used to help determine the validity of using ATCA as a marker for verification of cyanide exposure. Other directions include mapping endogenous concentrations of ATCA in blood, animal tissues, and hair. Also, exposure of animals to cyanide while monitoring ATCA concentrations in blood and urine will be used to determine the difference between endogenous ATCA concentrations and ATCA concentrations after exposure to cyanide.

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